IMMUNOPATHOLOGIC OBSERVATIONS IN LIVER ANGIOSARCOMA

Enrique Espinosa, M.D.

Department of Pathology
University of Louisville School of Medicine
Louisville, Kentucky

I. INTRODUCTION

In hepatic fibrosis and angiosarcoma associated with vinyl chloride exposure of industrial workers, manifestations of the disease could not be detected in most cases until the process was far advanced (1). Normal values of liver function tests were reported in a case with significant vinyl chloride hepatic fibrosis (2), and only a small percentage of workers of a plant unit where seven cases of liver angiosarcoma were diagnosed had abnormal blood screening tests (3). Thus, conventional liver function tests do not appear to be sensitive indicators of vinyl chloride liver disease. Development of more sensitive methods for detecting the disease in early stages would be of great importance. An approach to this may be provided by immunologic studies. In such a study the question arises whether the fibrotic and angiosarcomatous livers contain antigens that are different from those present in normal tissue and whether such changes could stimulate an immunologic response. The purpose of this study was to search for antigenic changes in the angiosarcomatous tissue and to test for possible presence of an antibody response in the host.

II. PROCEDURES AND MATERIALS USED

A. Patients' Sera and Tissue Specimens

Serum samples from B.F. Goodrich Co. workers with histories of vinyl chloride exposure of several years included samples from two individuals with liver angiosarcoma, ten with liver dysfunction with fibrosis and ten with normal liver function tests. The patients with angiosarcoma died and the diagnosis was confirmed at autopsy and portions of tumor and neighboring liver tissues were obtained at autopsy. Patients with liver dysfunction with fibrosis included individuals with

abnormalities in liver function tests and fibrosis detected at biopsy. Tissues were also obtained from coroner's autopsies of healthy individuals a few hours after death by gunshot wounds.

B. Tissue Extracts and Antisera

Liver angiosarcoma and adjacent liver tissue and post-mortem tissues considered to be normal were frozen and stored at -70°C until used. Portions were cut, thawed and homogenized in 2-3 volumes of distilled water in a Potter-Elvehjem grinder in an ice bath until a smooth suspension was obtained. After centrifugation at 20,000 x G for 30 min, the supernatant fluid containing the aqueous extract was lyophilized. Albino rabbits were immunized with the tumor or normal liver extracts in Freund's complete adjuvant and sera collected and stored following procedures detailed elsewhere (4). Reaction of these immune sera with human serum or plasma was eliminated by absorption with 100 mg of lyophilized, pooled normal human serum /ml antiserum. Antisera were routinely absorbed in this manner prior to use. Additional absorption with tissue extracts was carried out with 100 mg lyophilized extract/ml antiserum. Absorptions followed a procedure described previously (5).

C. Treatment of Tissue Extracts

Enzymatic treatment of tissue extracts was carried out with Pronase and trypsin as previously described (6). Periodate oxidation was done according to Rajam et al. (7). Ammonium sulfate and cold ethanol fractionations were carried out as detailed (8).

D. Immunodiffusion and Immunofluorescence

Double immunodiffusion was carried out in 0.8% agarose in phosphate-buffered saline pH 7.2 (PBS) containing 0.1% sodium azide. Circular wells, 2 mm in diameter, 3 mm apart were used. Immunoelectrophoresis was performed according to Scheidegger (9) using 0.8% agarose in 0.025 M Veronal buffer at pH 8.2.

In immunofluorescent studies cryostat sections of rat kidney, stomach or intestine and liver (4 microns) were used as substrate for antimitochondrial, antismooth muscle and antinuclear antibodies. Liver sections from rats exposed 4, 8 and 14 days to 1-2% vinyl chloride for 4 hr/day were also used. The sections were covered with dilutions of patients' sera for 45 min at room temperature, washed twice in PBS for 10 min and then covered with fluorescein conjugated IgG fraction of rabbit anti-human immunoglobulins serum (Cappel Laboratories, Inc.) for 45 min and washed as before prior to examination. Cryostat sections of liver angiosarcoma and liver tissue considered to be normal (4 microns) were washed twice in PBS for 10 min to wash off nonfixed immunoglobulins. After drying,

sections were stained with fluorescein conjugated IgG fractions of rabbit anti-human IgG serum and goat anti-human IgM serum (Cappel Laboratories, Inc.). Sections were washed as above and examined under the fluorescent microscope. Representative frozen sections were stained with hematoxylin and eosin to allow correlation between immunofluorescence and the histological findings. The antigenic preservation of the tissues was indicated by the demonstration of their staining by antinuclear factor according to the indirect immunofluorescent procedure.

E. Elution of Tumor-bound IgG

The tumor and liver tissues were extracted five times with PBS to wash off nonfixed immunoglobulins and then extracted at pH 2.5 to release bound IgG according to a procedure applied in the elution of renal-bound antibody (10).

F. Circulating Tissue Antigens

Liver-specific antigen LSA (8), tissue antigens of wide organ distribution (4) and bile antigens (11) were tested in the patients' sera by immunodiffusion as described previously.

III. RESULTS

A. Angiosarcoma-related Antigen

To test for presence of new antigens appearing in liver angiosarcoma, antiangiosarcoma serum was absorbed with human serum and liver extract and tested by immunodiffusion with extracts of both normal liver and angiosarcoma tumor at varying concentrations. This absorption eliminated all reactivity with liver extracts prepared from five normal individuals but not with the angiosarcoma extracts where one line of precipitation remained (Fig. 1). This line of precipitation could still be seen after additional absorption of the antiserum with kidney extract. In contrast, absorption with the tumor extracts eliminated completely the angiosarcoma-related line of precipitation. Absorption with spleen and lung extracts also eliminated this line of precipitation. Thus, the antigen appeared to be of restricted tissue distribution and not angiosarcoma specific. The antigen was inactivated by trypsin and Pronase and thus appeared to be a protein or closely associated to protein. Incubation of the tumor extracts for 1 hr at 4°C in citrate buffer, pH 2.5, resulted in inactivation of the antigen whereas incubation in phosphate buffer, pH 5.0, neutral or alkaline pH up to pH 10.0, did not affect it. The antigen was shown to be relatively thermolabile. Incubation of the tumor extracts for 30 min in PBS at 25° and 56°C did not affect the antigen whereas incubation at 70°C and higher

completely inactivated it. The antigen precipitated mainly at 20-30% saturated ammonium sulfate and at ethanol concentrations of 30-70% (Table I).

B. Absence of a Normal Tissue Antigen in Angiosarcoma

Antiliver serum absorbed with human serum gave several arcs of precipitation in immunoelectrophoresis with extracts of normal liver and angiosarcoma tissue (Fig. 2a). These lines could not be seen following additional absorption of the antiserum with normal liver. In contrast, absorption with liver angiosarcoma extract failed to eliminate one of the arcs of precipitation (Fig. 2b). Thus, the tissue antigen related to this arc of precipitation appeared to be absent in the angiosarcomatous tissue whereas the antigens corresponding to the other arcs were present. The absent antigen in angiosarcoma was shown to be present in kidney and lung extracts in addition to liver by absorption and direct immunodiffusion tests. Physicochemical characterization studies indicated this antigen to be unaffected by Pronase and trypsin and inactivated by periodate treatment. The antigen was relatively thermostable withstanding incubation at 70°C for 30 min in PBS. The antigen was destroyed following incubation of the liver extract in citrate buffer at pH 2.5 or lower for 1 hr at 4°C; incubation at pH 5.0 or higher (up to pH 10.0) did not affect it. This antigen precipitated over a wide range of ammonium sulfate and ethanol concentrations (Table I).

C. Angiosarcoma-bound IgG

IgG fluorescent staining appeared in a linear pattern in the peripheral portion of the tumor cells suggesting in vivo binding by the tumor of the immunoglobulin. This staining is illustrated in Fig. 3. Fig. 4 shows the angiosarcomatous cells surrounding irregular vascular spaces. Relatively coarse, linear fluorescence was also present along some hepatic cords and strands of connective tissue. There was no evidence of IgM. Control post-mortem liver tissue did not show any significant fluorescence of bound IgG. Staining for IgG of the tumor sections did not change after several washings at pH 7.2 indicating that the IgG was firmly bound to the tumor. In contrast, sections showed marked diminution of staining after washing at pH 2.5. Elution of bound IgG from saline-extracted tumor homogenates was thus attempted at acid pH. With the five successive saline extractions the amount of saline soluble IgG gradually diminished to nondetectable levels; and at acid pH bound IgG was released from the homogenate. Similar treatment of liver homogenates did not demonstrate presence of bound IgG (Table II).

D. Circulating Autoantibodies and Tissue Antigens

Serum autoantibodies to nuclei, mitochondria and smooth muscle were negative in all patients examined. In addition,

serum from these patients did not show reactivity with liver from rats exposed to vinyl chloride. Liver-specific antigen LSA (8), bile antigens (11) and other tissue antigens (4) associated with liver damage were not detected in these patients.

IV. DISCUSSION

The immunologic characteristics of cancer have been under intense investigation during recent years, and antigenic differences between normal and malignant tissue are considered to be fundamental factors in the immunologic approach to cancer therapy and diagnosis. Liver angiosarcomatous tissue was thus analyzed in this work for presence of neoantigens, normal tissue antigens and tumor-bound immunoglobulins. Several normal tissue antigens were found by immunodiffusion to be present in the tumor, but one antigen of rather wide organ distribution was not detected. These findings are in agreement with observations in other tumors indicating that tumor cells contain many of the antigens of their original hosts and lack some normal tissue antigens. For example, immunohistochemical studies have shown the loss of kidney antigens in stilbestrol- and x-ray-induced kidney tumors (12), of skin antigen in 3-methylcholanthrene-induced mouse squamous cell carcinoma (13) and of certain muscle antigens in 20-methylcholanthrene-induced rat rabdomyosarcoma (14). By far the most extensively studied class of tumors are the chemically induced hepatomata where deletion of liver antigens have been shown in tumors induced with 4-dimethylaminoazobenzene (15, 16), diethylnitrosamine (15) and 2-acetomidofluorene in the rat (15, 17) and o-aminoazotoluene in the mouse (18). In human carcinoma, loss of antigens have been reported in squamous cell carcinoma (12, 19), loss of the ABH blood group isoantigens in some solid tumors (20, 21) and of HL-A isoantigen in lymphoma (22). In addition, it has been well documented that as cells transform from a normal state to malignancy they may gain new antigenic specificities. Tumor-specific transplantation antigens have been demonstrated in a number of experimentally induced tumors (23-26) as well as in spontaneous tumors in man (27-29). In the present report immunodiffusion analyses of liver angiosarcoma and other human tissues with rabbit antiangiosarcoma serum did not indicate the presence of a tumor-specific antigen but rather of an antigen found in lung and spleen but not in liver and kidney. This antigen is being further characterized in our laboratory.

Of particular interest is the demonstration of tumor-bound IgG by immunofluorescence and elution experiments. This finding must however be interpreted with caution and should be confirmed in biopsy specimens. The tumor-bound IgG may represent specific antitumor antibody, antibody fixed by the tumor tissue "nonspecifically" or part of both. Further speculation is premature until it has been shown that the staining pattern

is due to the deposition of a specific antibody, that the eluted antibody is specific or until the relevant antigen has been identified. Work is in progress to determine the precise significance of the finding of IgG in the tumor.

V. SUMMARY

Immunodiffusion analyses of human liver angiosarcoma associated with vinyl chloride exposure indicated presence in the tumor of an antigen not detected in normal liver and kidney but found to be present in lung and spleen. This antigen was shown to be a protein, inactivated by Pronase and trypsin, relatively susceptible to heating and to acid pH and precipitated mainly at 20-30% saturated ammonium sulfate and at 30-70% ethanol concentrations. The tumor was shown to contain several antigenic constituents of normal tissue but one normal tissue antigen was not detected. This antigen was characterized as a substance unaffected by Pronase and trypsin and inactivated by periodate. It was relatively thermostable, affected by acid pH and precipitated over a wide range of ammonium sulfate and ethanol concentrations. Tumor specimens obtained at autopsy contained bound IgG as shown by immunofluorescence and elution experiments suggesting possible *in vivo* binding of IgG to the tumor.

Acknowledgements: The author wishes to thank Drs. W. M. Christopherson, G. R. Schrodt and P. H. Carstens for fruitful discussion and advice about the histologic sections and Drs. C. Tamburro and L. Maak for providing serum samples and autopsy material. I also wish to thank Miss M. VanBraun and Mrs. V. Petrey for skilled technical assistance. This work was supported in part by a grant from B. F. Goodrich, Co.

TABLE I. Angiosarcoma-related Antigen and Antigen Absent from the Tumor in Ammonium Sulfate and Ethanol Fractions

Fraction tested	Presence of[a]	
	Angiosarcoma-related antigen[b]	Antigen absent from Angiosarcoma[c]
Ammonium sulfate:		
0-20% saturation	+	-
20-30% saturation	++	+
30-50% saturation	-	+++
50-70% saturation	-	++
Ethanol:		
0-20%	-	++
20-30%	-	+++
30-50%	++	+++
50-70%	++	++
SN[d]	-	+

[a] +++, ++, + indicate strength of double diffusion reaction in dilution assay.

[b] Detected in angiosarcoma fractions.

[c] Detected in liver fractions.

[d] SN = supernate of the 70% ethanol precipitation, dialyzed and lyophilized.

TABLE II. IgG in Saline and Acid Extracts of Angiosarcoma and Liver Tissues

Preparation tested	Weight solid extracted from 1 gm (wet weight) tissue (mg)	Presence of IgG[a]
Angiosarcoma:		
Saline extract 1	29.8	+++
Saline extract 2	8.6	++
Saline extract 3	6.2	+
Saline extract 4	5.6	-
Saline extract 5	6.1	-
Acid extract	6.1	++
Liver:		
Saline extract 1	47.4	+++
Saline extract 2	14.5	+++
Saline extract 3	8.6	+
Saline extract 4	7.4	-
Saline extract 5	6.4	-
Acid extract	9.0	-

[a] Tested by immunodiffusion at concentrations of the eluates ranging up to 2%. Present at concentrations 0.05-0.1% (+++); 0.2-0.5% (++); 1-2% (+); negative at 2% (-).

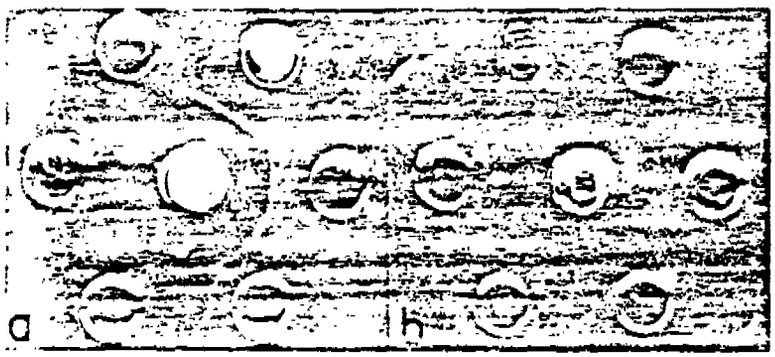

FIG. 1. Demonstration of angiosarcoma-related antigen. Peripheral wells have 2-fold serial dilutions of liver angiosarcoma extract (a) and normal liver extract (b). Dilutions are clockwise and start at 100 mg/ml in the upper right well. Central wells in each plate contain rabbit antiangiosarcoma serum absorbed with normal human serum and liver extract.

FIG. 2. Demonstration of tissue antigen absent in angiosarcoma. (a) Trough contains rabbit antihuman liver serum absorbed with normal human serum. (b) Trough contains the antiliver serum additionally absorbed with angiosarcoma extract. In both plates top wells have 10% solution of liver extract and lower wells angiosarcoma extract. Anode is to the right.

FIG. 3. Immunofluorescent staining of liver angiosarcoma by fluorescein conjugated IgG fraction of rabbit antihuman IgG serum (x 400).